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Bone fragility beyond strength and mineral density: Raman spectroscopy predicts femoral fracture toughness in a murine model of rheumatoid arthritis

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ABSTRACT

Clinical prediction of bone fracture risk primarily relies on measures of bone mineral density (BMD). BMD is strongly correlated with bone strength, but strength is independent of fracture toughness, which refers to the bone's resistance to crack initiation and propagation. In that sense, fracture toughness is more relevant to assessing fragility-related fracture risk, independent of trauma. We hypothesized that bone biochemistry, determined by Raman spectroscopy, predicts bone fracture toughness better than BMD. This hypothesis was tested in tumor necrosis factor-transgenic mice (TNF-tg), which develop inflammatory-erosive arthritis and osteoporosis. The left femurs of TNF-tg and wild type (WT) littermates were measured with Raman spectroscopy and micro-computed tomography. Fracture toughness was assessed by cutting a sharp notch into the anterior surface of the femoral mid-diaphysis and propagating the crack under 3 point bending. Femoral fracture toughness of TNF-tg mice was significantly reduced compared to WT controls ($p=0.04$). A Raman spectrum-based prediction model of fracture toughness was generated by partial least squares regression (PLSR). Raman spectrum PLSR analysis produced strong predictions of fracture toughness, while BMD was not significantly correlated and produced very weak predictions. Raman spectral components associated with mineralization quality and bone collagen were strongly leveraged in predicting fracture toughness, reiterating the limitations of mineralization density alone.

1. Introduction

Bone fragility fractures that occur in the absence of significant trauma are often associated with primary or secondary osteoporosis, and can result in serious patient morbidity and mortality (Johnell et al., 2004). Therefore, it is necessary to accurately identify high risk patients who require intervention, without imposing the unnecessary costs that could result from false positive classifications. Currently, individualized prediction of bone fracture risk primarily relies on measures of bone mineral density (BMD), often determined through dual energy X-ray absorptiometry (DXA), as well as clinical risk factors such as smoking, glucocorticoid treatment, and rheumatoid arthritis (RA) (Kanis et al., 2008; van Staa et al., 2006, 2003). DXA bone scans provide a *T*-score for anatomic sites of interest, such as the hip and spine, that describes the patient's deviation in areal BMD from an average young adult of the same gender and ethnicity. For women, the World Health Organization defines osteoporosis as a *T*-score below −2.5 (Kanis et al., 1994; WHO, 1994). When this standard definition of osteoporosis was used to classify female patients at high risk of bone fracture in a sample population, the sensitivity of fracture prediction was only 22.4% (Tremollieres et al., 2010). This evidence underscores the critical need for improved technologies and prediction modalities that will enable more sensitive and specific bone fracture risk predictions.

BMD is controversial as a predictor of fracture risk (Burger et al., 1999; Cranney et al., 2007; Cummings et al., 1993, 2006; De Laet et al., 1998; Siris et al., 2004; Stone et al., 2003; van Staa et al., 2003). The complex composition and structure underlying the mechanical integrity of bone likely explains the limitation of DXA bone scans to accurately predict fracture susceptibility.

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Indeed, ex vivo biomechanical studies have reported significant correlations between BMD and whole bone strength in a variety of loading modalities (Huber et al., 2008; Nielsen et al., 2007; Perilli et al., 2012). However, these strength-based measures do not account for bone's fatigue response or fracture toughness, which are important considerations in defining bone quality and predicting fragility fracture risk. Fracture toughness is generally independent of strength and refers to bone's ability to resist fracture by dissipating high local stresses associated with crack initiation and propagation (Ritchie, 2011). Bone is a complex heterogeneous and anisotropic composite of mineral (primarily calcium phosphate) and organic (primarily collagen) matrix with structural organization spanning multiple size scales. Within lamellar bone, collagen microfibrils are organized in oriented arrays that form the foundational substructure of bone. Hydroxyapatite-like mineral crystals, which are often calcium-deficient and contain carbonate and other substitutions compared to stoichiometric hydroxyapatite, are deposited within and around this collagen matrix (Fratzl et al., 2004; Glimcher, 1984; Landis et al., 1996). The toughness of bone derives from the micro- and nano-structural organization as well as reinforcement to the brittle mineral phase by the underlying collagen matrix. The interested reader is referred to excellent reviews on strength versus fracture toughness (Ritchie, 2011) and the toughness of bone (Launey et al., 2010; Ritchie, 2010; Zimmermann et al., 2012) for more details.

Pathological changes that occur with natural aging and through a variety of disease processes lead to compositional and structural changes in the bone, including loss of mineral mass in osteoporosis (Kanis et al., 1994), non-enzymatic glycation of collagen in type 1 diabetes (Saito and Marumo, 2010; Silva et al., 2009; Tang and Vashishth, 2011; Vashishth, 2007), or concomitant deterioration in the mineral and organic phases of the bone matrix in inflammatory diseases such as RA (Abdulghani et al., 2009; David and Schett, 2010; Lacativa and Farias, 2010; Li and Schwarz, 2003; Romas, 2005; Takahata et al., 2012). These changes invariably affect bone strength and toughness, which cannot be noninvasively measured or reliably predicted with current clinical technologies.

There is a critical need for improved diagnostic modalities to detect compositional changes in bone and predict the strength-independent functional properties such as fracture toughness. Raman spectroscopy is a promising technology that can produce unique information about the compositional biochemistry of bone by measuring light scattering that results from molecular vibrations, with spectral shifting that corresponds to the unique vibrational energy levels within each type of molecule. Pathological changes in matrix components, including phosphate and carbonate as well as the amide backbone of collagen, can be detected by Raman spectroscopy, but not through currently available clinical tools (Carden and Morris, 2000). Therefore, we hypothesized that Raman spectroscopy would predict pathological changes to cortical bone fracture toughness while BMD alone would not provide accurate predictions. This hypothesis was tested using a transgenic murine model of RA, which constitutively overexpresses the inflammatory cytokine tumor necrosis factor-alpha (TNF-α) and develops secondary osteoporosis.

2. Methods

2.1. Animals and tissue processing

All animal studies were performed in accordance with protocols approved by the University of Rochester's Committee on Animal Resources. TNF-transgenic (TNF-tg) mice (line 3647 in a C57BL/6J background) (Keffer et al., 1991) and wild type (WT) C57BL/6J mice were used in this study ($n=7$ per group). The TNF-tg mice serve as a model of RA and invariably develop chronic inflammation and erosive arthritis with secondary osteoporosis by five months of age (Proulx et al., 2007; Takahata et al., 2012). The left femurs of male mice from each genotype in the age range of 20–22 weeks were collected for imaging and biomechanical testing. Each bone was cleaned and stored at −80 °C until the day of micro-computed tomography (micro-CT) and Raman spectroscopic measurements, as well as subsequent biomechanical testing.

2.2. Micro-computed tomography

The left femur of each mouse was scanned by micro-CT (VivaCT 40; Scanco Medical; Bassersdorf, Switzerland), at a 10.5-micron isotropic resolution using an integration time of 300 ms, energy of 55 kVp, and intensity of 145 μA. The cortical bone mineral density (BMD), cortical thickness, and area moment of inertia about the major cross-sectional axis were measured by averaging over a region of 300 μm (28 slices) at the femoral mid-shaft.

2.3. Raman spectroscopy

Following micro-CT, Raman spectra were acquired from the medial mid-diaphysis of the same femurs over a 3.5 mm^2 area using a locally constructed Raman spectroscopy system (Maher et al., 2011). All spectra were truncated to the 910–1740 cm^{-1} region, which includes many relevant mineral and organic matrix bands (Carden and Morris, 2000), and normalized to their mean absolute deviations. Commonly studied component ratios were calculated, such as the phosphate mineral-to-matrix ratio (PMMR) based on the phosphate ν_1 (924–986 cm^{-1}) and amide I (1596–1730 cm^{-1}) areas, phosphate-to-carbonate ratio (PCR) based on the phosphate ν_1 and carbonate ν_1 (1057–1098 cm^{-1}) areas, carbonate mineral-to-matrix ratio (CMMR), and the amide I 1660 cm^{-1}/1690 cm^{-1} ratio. Peak areas were calculated by summing the Raman intensity in the spectral regions listed above. The full width half maximal (FWHM) bandwidth of the phosphate ν_1 peak, which is related to mineral crystallinity (Morris and Mandair, 2011; Penel et al., 1998), was calculated by fitting with a single Gaussian curve.

2.4. Femoral fracture toughness

Fracture toughness of the same femoral mid-diaphysis was subsequently measured by methods adapted from Ritchie et al. (2008). Specifically, a razor sharp circumferential through-wall notch was cut into the anterior surface of the femoral diaphysis. A starter notch was cut with a 0.22 mm gigli saw (RISystem; Davos, Switzerland), which was then sharpened using a razor blade under irrigation with a 1 μm diamond suspension. The notches were cut to relative depths to produce half-notch angles of $75^\circ \pm 4.1^\circ$ (Fig. 1h). The anterior surface was chosen for notching based on findings suggesting that physiological loading is primarily tensile in the anterior quadrant of rat and mouse femora (Indrekvam et al., 1991; Ramasamy and Akkus, 2007; Vashishth, 2008). After notching, the bones were stored in PBS overnight at 4 °C and then allowed to warm to room temperature prior to three-point bending, which was conducted using an Instron 8841 DynaMight™ Axial Testing System (Instron Corp.; Canton, MA) with a 6 mm support span and a 50 N load cell. Bending tests were performed at a displacement rate of 0.001 mm/s until failure (fast fracture) with the femurs submerged in PBS and the anterior, notched side of the bone in tension. A stereomicroscope was used to ensure that the loading post was aligned precisely in plane with the notch (Fig. 1a,b).

After testing, bone specimens were fixed, dehydrated, and gold sputter coated for scanning electron micrograph (SEM; LEO 982 FE-SEM; Carl Zeiss SMT; Thornwood, NY) imaging to assess the extent of sub-critical (stable) crack growth prior to fracture. The half-crack angle, average cortical thickness, and mean radii were measured from backscattered SEM images of the fracture surfaces using custom algorithms written in MATLAB (R2011a; MathWorks Inc.; Natick, MA). Micro-CT based measures of mean cortical thickness around the full bone circumference were not used because the anterior quadrant, once notched, does not contribute to the load response during testing and is generally thicker, which would bias the measurements. These data along with the fracture load and support span were used to estimate the critical stress intensity factor (K_C; fracture toughness) for each specimen (Ritchie et al., 2008) according to:

$$K_C = F_b \frac{P_f S R_o}{\pi\left(R_o^4 - R_i^4\right)} \sqrt{\pi R_m \theta_C}$$

where P_f is the load at fracture, S is the span length, R_o and R_i are the mean outer and inner radii, R_m is the average of R_o and R_i, θ_C is the half-crack angle at fracture